PB80-217722

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(U.S.) Corvallis Environmental Research Lab.
College, AK

1978

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BIBLIOGRAPHIC INFORMATION

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PERFORMER: Corvallis Environmental Research Lab., College, Alaska. Arctic Environmental Research Station. EPA-600/J-78-173

Text in English; summary in French. Pub. in ARCTIC, v31 n3 p324-338 Sep 78. Prepared in cooperation with Alaska Univ., Fairbanks. Inst. of Water Resources.

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KEYWORDS: *Microorganisms, *Ecology, *Oil pollution.

Available from the National Technical Information Service, Springfield, Va. 22161

PRICE CODE: PC A02/MF A01

EPA-600/J-78-173
JOURNAL ARTICLE

PB80-217722

ARCTIC
VOL. 31, NO. 3 (SEPT. 1978), P. 324-338

Response of Microorganisms to Hot Crude Oil Spills on a Subarctic Taiga Soil

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ABSTRAC . This study was conducted on the short-term effects of seasonal spills of hot Prudhoe Bay crude oil on microorganisms in a taiga soil in interior Alaska. Following a winter spill, the filamentous fungal populations were inhibited whereas the heterotrophic bacterial populations were stimulated. After a summer spill there was an initial depression of both the filamentous fungal and bacterial populations followed by a general enhancement. In both oil spill plots, yeasts; along with the denitrifying, proteolytic, oil-utilizing, and cellulose-utilizing microorganisms; were favorably affected by the oil. Soil respiration was also enhanced in the oiled plots. An extended period of study is required to fully evaluate the impact of oil on the soil microflora and the role of these micoorganisms in recovery of oil-inundated areas in subarctic ecosystems.

RÉSUMÉ. Cette étude était effectuée sur les effets, à court terme, d'écoulements saisonniers de brut chaud de Prudhoe Bay, sur les micro-organismes dans un sol de Taiga de l'Alaska de l'intérieur. A la suite d'un écoulement hivernal, les populations de mycetes filamenteux étaient imbibées, alors que les populations bactérielles hétérotrophiques étaient stimulées. A la suite d'un écoulement estival, il y avait initialement une diminution à la fois des populations de mycetes filamenteux et de bacteries puis une reprise générale dans les deux terrains à écoulement d'huile; avec la dénitrification et les micro-organismes protéolytiques utilisant le pétrole et la cellulose, les levures étaient favorablement affectées par le pétrole. La respiration du sol était aussi stimulée dans les terrains pollués. Il faudra une longue période pour évaluer pleinement l'impact du pétrole sur la microflore du sol et le rôle de ces micro-organismes dans la récupération des régions inondées de pétrole dans le système écologique subarctique.

Traduit par Alain de Vendigies, Aquitaine Co. of Canada Ltd.

INTRODUCTION

Microorganisms are important in the cycling of energy and nutrients as well as in degradation of pollutants in ecosystems. Oil utilization by microorganisms has been demonstrated in numerous studies. However, comparatively little attention has been given to the effects of crude oil on natural microbial populations, especially those in arctic and subarctic terrestrial ecosystems. Of the few terrestrial studies which have been conducted in arctic and subarctic environments, oil has been reported to stimulate the growth of filamentous fungi, yeasts, heterotrophic bacteria, and oil-utilizing microorganisms (Scarborough and Flanagan, 1973; Sexstone and Atlas, 1977; Gossen and Parkinson, 1973; Campbell *et al.*, 1973). Increased respiration rates (Gossen and Parkinson, 1973; Campbell *et al.*, 1973), and increased denitrification but decreased nitrification potential (Lindholm and Norrell, 1973) have been

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reported in oiled soils. Results of laboratory studies using C^{14} —cellulose powder as test substrate indicated a stimulatory effect of oil on cellulose decomposition whereas the converse was found in field studies using filter paper as test substrate (Parkinson *et al.*, 1975). The addition of fertilizer to oiled soils stimulated bacterial numbers and accelerated utilization of the n-saturate oil fraction (Jobson *et al.*, 1974) as well as increasing respiration rates (Hunt *et al.*, 1973). In these studies, unheated oil was applied in the summer. In our study, hot crude oil was applied on a subarctic taiga soil in both winter and summer to determine the effects of seasonal spills of hot crude oil on soil microbial populations and their activities.

This paper reports on the short term effects of hot crude oil spills on heterotrophic bacteria, filamentous fungi and yeasts, anaerobic, denitrifying, proteolytic, cellulose-utilizing and oil-utilizing microorganisms, and soil respiration. In previous terrestrial oil studies, the response of anaerobic, denitrifying, proteolytic and cellulose-utilizing bacteria and cellulose-utilizing fungi have not been examined.

This research is part of the Cold Regions Research and Engineering Laboratory project studying the fate and effects of simulated large scale hot oil spills on permafrost underlain terrain in interior Alaska.

MATERIALS AND METHODS

Study site

The experimental plots are located in the Caribou-Poker Creeks Research Watershed in interior Alaska. The soil, a Saulich silt loam, is classified as a histic pergelic cryaquept (Reiger *et al.*, 1972). A detailed description of the location, terrain and vegetation of the study is presented elsewhere (Jenkins *et al.*, this volume).

Three study plots, each 10 m x 50 m with the long axis downslope, were designated. One plot was the control (unoiled plot), another was the site of the winter oil spill (winter plot) and the third was the site of the summer oil spill (summer plot). The control plot was located between the two oil plots. Hot (57 °C) Prudhoe Bay crude oil (7570 l) was applied to the winter plot in February and to the summer plot in July. The characteristics of the crude oil and the procedure used for conducting the spills are described by Jenkins *et al.* (this volume).

Collection and processing of soil samples

Soil samples were collected from the A_1 horizon (zone of organic matter accumulation) and from the upper 5-7 cm of the C^2 horizon in each test plot. Soil sampling in the oiled plots was confined to the first 5 to 10 m from the line of oil application, where the concentration of oil appeared to be highest. The soil sampling dates were 27 February, 17 June, 14 July, 12 August, and 29 September, 1976. Each oiled plot was first sampled within 24 hours after oil was first applied. During the winter sampling (February) a gasoline-powered

corer was used to obtain soil cores (frozen). During the plant growing season (June to September) soil samples were taken with a hand-operated corer. Several random soil cores were taken from each plot. Soil cores were visually examined and then separated into A_1 and C_2 sections and placed in separate containers. The soil cores were kept at 4 °C and processed within 24 hours after sampling. During sample processing, plant roots were removed, the soil cores were composited and subsamples were taken for microbial and chemical analyses. All dilutions and plating for microbial analyses were done in a 10 °C environmental room to minimize possible heat injury to psychrophilic microorganisms.

Enumeration of microorganisms

Changes in microbial numbers were monitored using dilution plate count (surface-spread) and Most-Probable-Number (MPN) techniques. Plate count agar (Difco) with cycloheximide (50 mg/l) plus additional agar (5 g/l) was used for counting heterotrophic and anaerobic bacteria. Martin's medium (Martin, 1950) was used for enumerating filamentous fungi and yeasts. A gelatin medium and the procedure described by Rodina (1972) was used to count proteolytic bacteria. A nitrate medium and a five-tube MPN procedure (Alexander, 1965) was employed to determine the abundance of denitrifying bacteria. Five replicate plates or five tubes per dilution were used for microbial enumeration except for the anaerobes which were done in triplicate. Incubation at 20 °C for one week and 4 °C for two weeks was used for all groups except the denitrifiers which were incubated for one month at both temperatures. Anaerobiosis for the determination of the abundance of anaerobic bacteria was attained by evacuating Brewer jars, flushing with nitrogen gas containing 5% carbon dioxide and using copper sulfate treated steel wool (Parker, 1955).

The enumeration of oil-utilizing microorganisms was a two-step process using a five-plate MPN (Most-Probable-Number) procedure. Initially, samples were plated on a modified silica gel medium containing 1% Prudhoe Bay crude oil. After one month incubation (at both 4 °C and 20 °C), these plates were replicated onto plate count agar to estimate the bacterial population and onto Martin's medium to estimate the fungal populations. The replica plates were subsequently incubated, one week for the 20 °C plates and two weeks for the 4 °C plates. The presence of one or more colonies on the replica plate constituted a positive test.

The silica gel plates were prepared according to the procedure of Funk and Krulwich (1964) with the mineral salts medium of Bushnell and Haas (1941). Our modification was the addition of a sufficient quantity of colloidal silica (Cab-O-Sil MS obtained from Cabot Corp., Boston, Mass.) to the mineral medium to make a final concentration of 1% colloidal silica in the gel plates. The colloidal silica was added to help maintain dispersion of oil in the gel plates. In making the silica gel plates, the components were autoclaved separately and allowed to come to room temperature before use. Sterilized oil (Robertson *et al.*, 1973) was added to the mineral-colloidal silica medium and

the medium was mechanically mixed to disperse the oil. Then the other components were added, the mixture shaken and the plates poured. Gelling occurred within one minute, which was also important in maintaining dispersion of the oil in the plates.

The estimation of the numbers of cellulose-utilizing microorganisms was also done on silica gel plates using the five-plate MPN procedure. The silica gel plates for this determination contained 1% cellulose (Sigmacell — Type 20) as a sole carbon source and the colloidal silica was not added.

Soil respiration measurements

In vitro respiration rates of soil samples were determined by measuring carbon dioxide evolution rates in biometer flasks (Bellco Glass, Inc.) according to the procedure of Johnson and Curl (1972). Five replicate determinations were done on samples from each soil horizon in each test plot with incubation at 4 °C and 20 °C.

In situ soil respiration was determined by measuring carbon dioxide evolution rates in the field. Open-ended cylinders (7.5 cm diameter) were sunk into the test plots. In the control plot, the live moss plant layer was clipped before the cylinders (10) were embedded to ensure that only litter, root and soil respiration were being measured. In the oiled plots, two sets of cylinders were embedded, one with the killed moss layer removed (5 cylinders per plot) and one with the killed moss layer intact (10 cylinders per plot). Carbon dioxide evolution rates were determined according to Coleman (1973), with the exception that the alkali jars were suspended (from the rubber stoppers used to cap the cylinders) instead of being placed on the soil surface. Control cylinders containing only jars of alkali were run simultaneously during the 24 hour-period that respiration rates were being measured.

Soil water, pH and oil determination

Water content of soil samples was determined using the gravimetric method described by Gardner (1965). Soil pH of fresh soil samples was measured using a glass electrode in a 1:1 soil-distilled water suspension (Peech, 1965).

Oil content in soil samples was determined based on gravimetric analysis of benzene extracts of soil. Three replicates of approximately 15 g wet soil from each plot at each soil depth were dried at 105 °C for 24—48 hr. Each replicate soil sample was extracted 4 to 5 times with 50 ml portions of benzene. The extracts were combined and evaporated to dryness in a fume hood at room temperature and the residue weighed. The percent dry residue obtained from the unoiled soil was subtracted from the percent dry residue from oiled soil to give corrected percent oil from oiled plots. The weight of the oil contributing to the dry weight of soil was taken into consideration in the calculation of the oven dry weight of oiled soils.

Statistical analyses

Counts of microorganisms and *in vitro* soil respiration rates are expressed on the basis of oven dry weight of soil. Plate counts and soil respiration rates

(untransformed data) were statistically analyzed using analysis of variance and Duncan's multiple range test (Duncan, 1955). Confidence limits were calculated for the MPN counts. For the comparison of two MPN estimates, non-overlap of the 95% confidence limits was considered indicative of a significant difference between the estimates (Cochran, 1950).

RESULTS AND DISCUSSION

A preliminary examination of the data suggested that the microbial populations and their activities showed similar response trends at both 4 °C and 20 °C incubation temperatures. Also, respiratory activity and some microbial populations were stimulated to a greater extent at 4 °C than at 20 °C. Thus, only the 4°C data are presented here since 4°C more closely approximates the soil temperature in the research watershed areas where our test plots are located (Slaughter, C., unpublished data). The dilution plate count method and MPN enumeration procedures used in this study, despite their limitations, are sensitive to real differences in microbial counts among the test plots or within a plot over a period of time, because it is the change in value that is important rather than the absolute value.

The effect of oil application on heterotropic bacterial counts is shown in Table 1. Beginning immediately after the spill in the winter plot, the numbers

TABLE 1. Heterotrophic bacterial counts (x 10^6/g soil) in oiled and unoiled (control) plots

Sampling time	Control plot	Winter plot	Summer plot
	A_1 Horizon		
February[a]	2.8	5.4**	—
June	6.8	120**	—
July[b]	3.9	430**	2.8*
August	2.4	170**	340**
September	0.7	210**	640**
	C_2 Horizon		
February[a]	1.2	1.8*	—
June	2.2	2.1	—
July[b]	0.9	41**	0.6**
August	1.4	14**	25**
September	0.3	12**	210**

[a]Within 24 hours after winter oil spill.
Within 24 hours after summer oil spill.
*Significantly different from the control at 5% level.
**Significantly different from the control at 1% level.

TABLE 2. Filamentous fungal propagule counts (x 10^4/g soil) in oiled and unoiled (control) plots

Sampling time	Control plot	Winter plot	Summer plot
	A_1 Horizon		
February[a]	130	92*	—
June	120	14**	—
July[b]	120	18**	49**
August	34	23**	31*
September	24	10**	100**
	C_2 Horizon		
February[a]	19	4**	—
June	24	0.7**	—
July[b]	13	8**	17**
August	14	4**	5**
September	5	2**	7*

[a]Within 24 hours after winter oil spill.
[b]Within 24 hours after summer oil spill.
*Significantly different from the control at 5% level.
**Significantly different from the control at 1% level.

of heterotrophic bacteria were increased relative to the control plot, and the increased counts persisted into September. In the summer plot, after an initial depresssion of the heterotrophic bacterial counts, there was a continual increase in numbers with the highest counts occurring in September. The increases in bacterial numbers in both oiled plots were significant relative to the control plot indicating a stimultory effect of oil on the heterotrophic bacterial population, the extent of stimulation being greater in the summer plot. Our findings on the response of heterotrophic bacterial populations to oil agree with those of Sextone and Atlas (1977).

It is evident that there is a differential response of the filamentous fungal population to seasonal oil spills. Numbers of filamentous fungal propagules (Table 2) were reduced in both soil horizons immediately following the winter spill and remained so throughout the plant growing season. The immediate impact of the summer spill was to reduce fungal counts in the A_1 horizon, but by September fungal counts were five times higher than the control. In the C_2 horizon there was an overall increase in counts with an apparent reduction occurring in August. The inhibitory effect of oil on fungal populations (enumerated by the dilution plate method) were similarly observed by other workers (Miller *et al.*, this volume; Antibus and Linkins, this volume), who used direct counting techniques to estimate the fungal populations. According to Griffiths and Siddiqi (1961) and Montégut (1960), the dilution plate counts

can be used as an indication of the fungal populations activity. However, Warcup (1967) suggested that, because of limitations, the dilution plate method may not necessarily correlate with fungal biomass or activity.

In both oiled plots, the counts of yeasts and proteolytic and anaerobic bacteria significantly increased above their initial levels (unpublished data); the September counts (Table 3) show the increases relative to the control plot. These significant increases suggest that an enhancement of these populations in the A_1 and C_2 horizons resulted from the oil spills.

The denitrifying bacterial population (Fig. 1), showed significant increases in both winter and summer plots relative to the control plot. This indicates a stimulatory effect of oil and suggests an increase in denitrification potential in the oil plots. This is consistent with the findings of Lindholm and Norrell (1973). The increased denitrification potential in the oiled soils points out the need for considering the type and timing of fertilizer applications if fertilizers are to be used to enhance recovery of oiled areas.

Cellulose-utilizing bacterial numbers (Fig. 2) increased in the winter plot relative to the control. In the summer plot, this population was initially unaffected, but by September significant increases in counts over the control occurred. On the other hand, numbers of cellulose-utilizing fungi (Fig. 3) remained comparable to the control in both oiled plots. Oil-utilizing bacteria (Fig. 4) and yeasts (Fig. 5) in the winter plot were significantly increased relative to the control. In the summer plot, counts of these oil-utilizing populations were initially comparable to the control but by September increases had occurred. However, counts of the oil-utilizing fungal populations in both oiled plots showed no significant changes relative to the control (Fig. 6). The significant increases in cellulose-utilizing, and oil-utilizing bacteria and oil-utilizing yeasts indicate an enhancing effect of oil

TABLE 3. September plate counts (x 10^5/g soil) in oiled and unoiled (control) plots

Microbial population	Control plot	Winter plot	Summer plot
	A_1 Horizon		
Yeasts	1	12**	590**
Proteolytic bacteria	1	120**	1100**
Anaerobic bacteria	4	36**	42**
	C_2 Horizon		
Yeasts	0.2	3**	290**
Proteolytic bacteria	0.2	5**	370**
Anaerobic bacteria	2	5**	10**

**Significantly different from the control at 1% level.

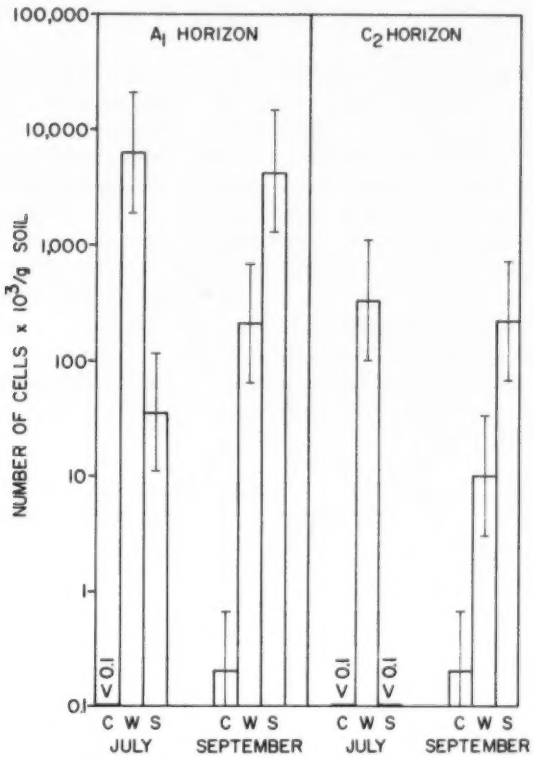

FIG. 1. MPN counts of denitrifying bacteria in oiled and unoiled (control) plots. C = Control Plot; W = Winter Plot; S = Summer Plot; Brackets represent 95% confidence intervals.

on these populations. There was no apparent effect of the oil on the cellulose-utilizing and oil-utilizing filamentous fungal populations.

The fact that some of the microbial populations functional in the carbon and nitrogen cycles have not been adversely affected suggests that nutrient transformations carried out by these populations are not eliminated. However, the actual activity of these functional groups is determined by a balance of the physiological requirements of the microorganisms and the existing environmental conditions.

The effect of oil on *in vitro* soil respiration rates is shown on Table 4. An immediate depression of soil respiration rates was apparent one day after the spill in both horizons of the winter plot and in the C_2 horizon of the summer plot. However, by the end of the plant growing season, the overall effect of oil on soil respiration rates was one of enhancement. The higher respiration rate in the A_1 horizon of the control plot in February relative to the other months is not too surprising in view of the evidence presented by Greenwood (1968)

that freezing and thawing of soil results in increased organic matter decomposition.

The effect of seasonal oil spills on *in situ* soil respiration is shown in Table 5. Soil respiration rates in cylinders with the killed moss layer removed were not significantly affected by the addition of oil. However, in cylinders with the killed moss layer intact, respiration rates were significantly increased in some cases. Although limited, these results suggest that the enhancing effect of oil on soil respiration *in situ* may be due to an increase in available substrate not only from the spilled oil but also from plants killed as a result of the oil spill. This needs further study. Respiration rates within a set of cylinders in the test plots were highly variable with the standard error ranging from 0.31 to 1.81. This high variation may be due to a methodology artifact or more likely due to the heterogeneity of the soil within each plot.

Because of oil damage on higher and lower plants shown by other studies (Hunt *et al.*, 1973; McCown *et al.*, 1973; Miller *et al.*, this volume; Antibus and

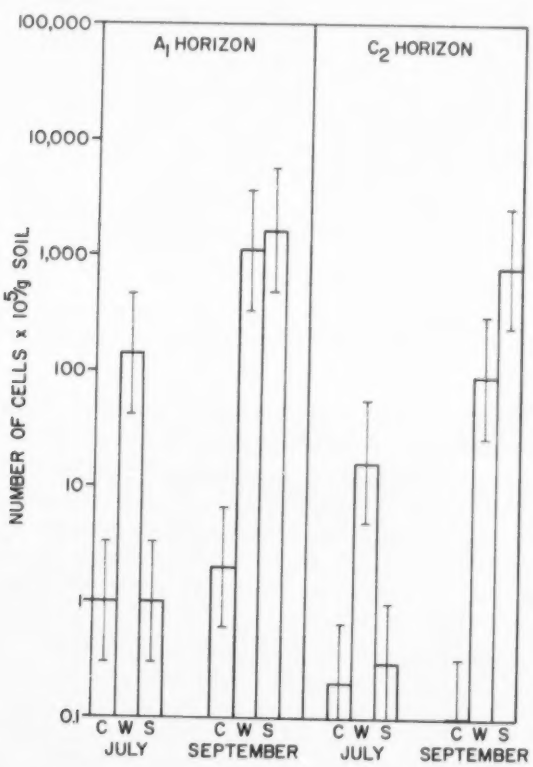

FIG. 2. MPN counts of cellulose-utilizing bacteria in oiled and unoiled (control) plots. C = Control Plot; W = Winter Plot; S = Summer Plot; Brackets represent 95% confidence intervals.

Linkins, this volume), the input of plant residues into the soil system in oil perturbed areas may be considerable. Jenkins *et al.*, (this volume) similarly observed damage to vegetation in our test plots, the damage being more extensive in the summer plot. This coincides with our finding of greater increases in microbial populations and soil respiration rates in the summer plot compared to the winter plot. This suggests that the increase in levels of soil microbial populations and activity may be due not only to the presence of petroleum substrate but also to the presence of substrates released from dying or dead plants.

Concentrations of benzene-extractable oil ranged from 4.3 to 17.9% in a A_1 horizon and from 0.1 to 1.5% in the C_2 horizon in the oiled plots. Although the concentration of oil was greater in the A_1 layer, the impact of oil was not limited to this horizon. Both A_1 and C_2 layers showed significant increases in

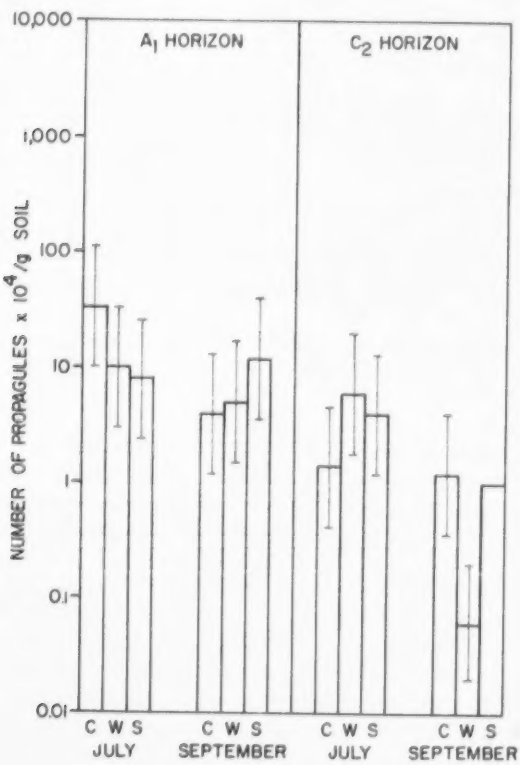

FIG. 3. MPN counts of cellulose-utilizing filamentous fungi in oiled and unoiled (control) plots. C = Control Plot; W = Winter Plot; S = Summer Plot; Brackets represent 95% confidence intervals.

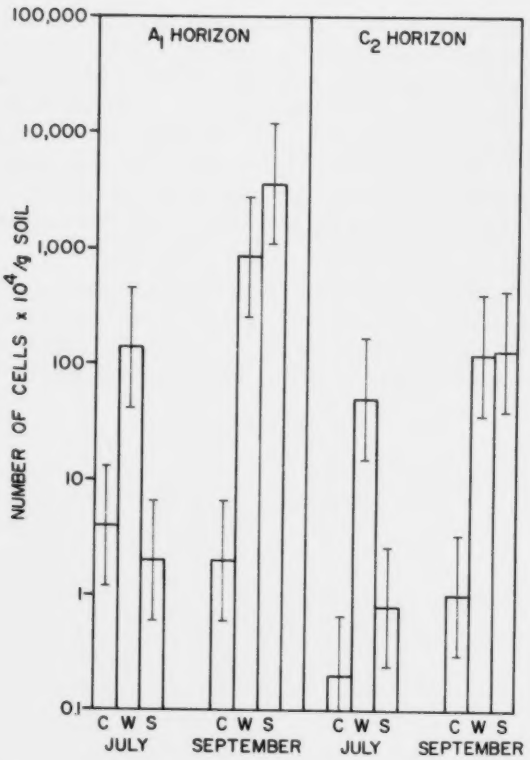

FIG. 4. MPN counts of oil-utilizing bacteria in oiled and unoiled (control) plots. C = Control Plot; W = Winter Plot; S = Summer Plot; Brackets represent 95% confidence intervals.

microbial populations and respiratory activity, and in the case of the filamentous fungal population, significant reductions. However, the response in the C_2 layer was sometimes slower to appear. In comparing the enhancing effect of oil in the two horizons, the extent was generally greater in the A_1 than in the C_2 layer.

The pH of soil sampled from the test plots showed a slight increase in the oiled plots relative to the control plot. The mean soil pH in the A_1 and C_2 horizons of the oiled plots was 5.8 and 5.5, respectively, whereas in the control plot it was 5.5 and 5.2. The soil pH in the control plot is similar to that reported by Rieger *et al.*, (1972) but is higher than reported by Jenkins *et al.* (this volume). The difference in soil pH can be attributed to the difference in the procedures used. We measured soil pH in fresh samples whereas Jenkins *et al.*, measured soil pH in oven dry samples.

In conclusion, the soil microbial populations showed a rapid and differential response during the first year after seasonal spills of hot crude oil. The

TABLE 4. *In vitro* soil respiration rates (mg CO_2/24 hr/50 g soil) in oiled and unoiled (control) plots

Sampling time	Control plot	Winter plot	Summer plot
	A_1 Horizon		
February[a]	13.6	5.6**	—
July[b]	4.0	8.3**	10.1**
August	4.3	7.8**	15.3**
September	1.9	7.8**	18.9**
	C_2 Horizon		
February[a]	1.3	n.d.[c]	—
July[b]	1.8	3.0**	0.6**
August	1.9	3.9**	3.1**
September	1.2	2.8*	6.8**

[a]Within 24 hours after winter oil spill.
[b]Within 24 hours after summer oil spill.
[c]Not detectable.
*Significantly different from the control at 5% level.
**Significantly different from the control at 1% level.

TABLE 5. *In situ* soil respiration (g CO_2/24 hr/m^2) in oiled and unoiled (control) plots

plot	Control plot	Winter plot		Summer plot	
	a	b	c	b	c
June 10	4.7	—	7.0	—	—
July 16	3.7	—	5.1	—	5.7
August 3	4.8	4.6	5.8	5.7	8.6*
August 12	2.2	3.3	4.8*	2.7	5.7**
September 1	2.9	1.6	4.0	4.5	6.4

[a]Respiration cylinders embedded after removal of live moss layer.
[b]Respiration cylinders embedded after removal of dead moss layer.
[c]Respiration cylinders embedded without removal of dead moss layer.
* Significantly different from control at 5% level.
**Significantly different from control at 1% level.

short-term effects ranged from inhibition to stimulation of microbial growth and respiratory activity. These response patterns are similar to those resulting from unheated oil applications. However, it is apparent that an extended period of study is required to fully evaluate the impact of hot oil spills on the soil microflora and the role of microorganisms in recovery of oil-inundated areas in subarctic environs.

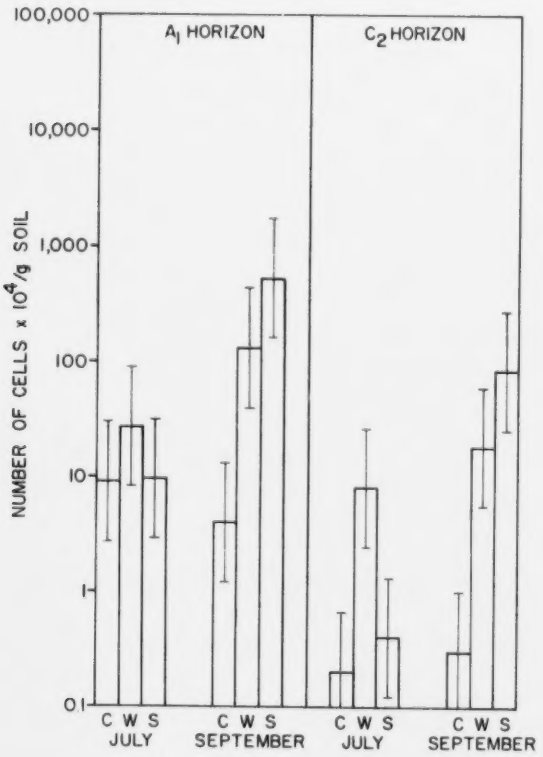

FIG. 5. MPN counts of oil-utilizing yeasts in oiled and unoiled (control) plots. C = Control Plot; W = Winter Plot; S = Summer Plot; Brackets represent 95% confidence intervals.

ACKNOWLEDGEMENTS

We wish to thank the U.S. Army Cold Regions Research and Engineering Laboratory and the Arctic Environmental Research Station, U.S. Environmental Protection Agency for financial and logistical support. We gratefully acknowledge the technical assistance of Ms. Joan Forshaug, Mrs. Ruth McFadden, and Mr. Robert Jackson.

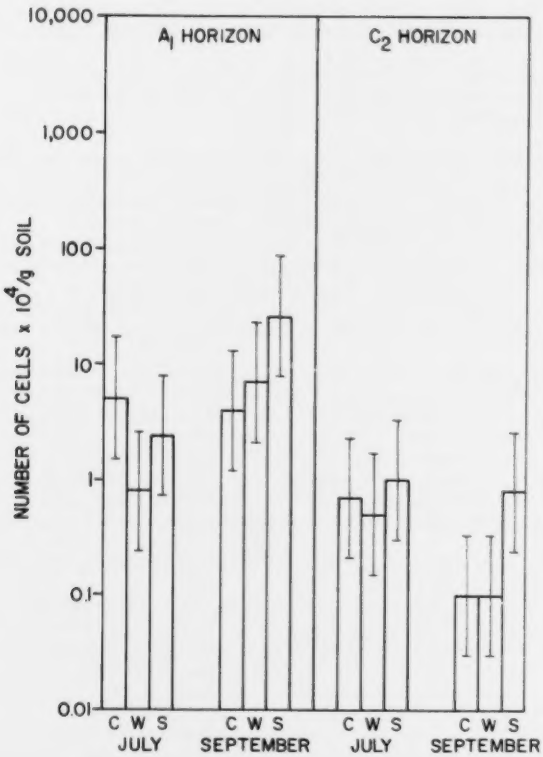

FIG. 6. MPN counts of oil-utilizing filamentous fungi in oiled and unoiled (control) plots. C = Control Plot; W = Winter Plot; S = Summer Plot; Brackets represent 95% confidence intervals.

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